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Relative Inactivation by *Staphylococcus aureus* of Eight Cephalosporin Antibiotics

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These studies extend the recent observation that cefazolin is inactivated to a greater extent than cephaloridine by some strains of penicillinase-producing *Staphylococcus aureus*, whereas cephalothin undergoes little if any inactivation. In Mueller-Hinton broth (inoculum, 3×10^6) 100 recently isolated strains had minimal inhibitory concentrations (MICs) ≤ 2 μg/ml for cephalothin and cephaloridine, whereas in Trypticase soy broth (TSB) 50% had MICs > 2 μg/ml and 10% (designated "resistant" strains) were >8 μg/ml for cephaloridine but remained ≤ 2 μg/ml for cephalothin. A large inoculum (3×10^7) of strains with high MICs in TSB almost completely inactivated 50 μg of cefazolin per ml in 6 h, with progressively less inactivation, in the following order, of cephaloridine, cephalexin, cephradine, cephapirin, and cefamandole; cefoxitin and cephalothin underwent little if any inactivation. The greater inactivation in TSB than in Mueller-Hinton broth appeared to be due to a greater production of β-lactamases by each colony-forming unit, since the inoculum size in the two broths was not significantly different. In contrast, "susceptible" strains (MICs ≤ 2 μg/ml in both broths) inactivated cephaloridine more than cefazolin, and equal amounts of powdered bacterial extracts confirmed the fact that qualitatively different β-lactamases were produced by the susceptible and resistant strains. Disk diffusion tests were unreliable in separating the two groups of staphylococci. The clinical significance of inactivation by strains with high MICs is not known but, unless susceptibility can be clearly established, cephalothin appears preferable for severe staphylococcal infections, since it undergoes little if any inactivation by any strains of staphylococci.

Benner et al. (2) showed in 1965 that 50% of strains of *Staphylococcus aureus* had minimal inhibitory concentrations (MICs) higher than 2 μg/ml for cephaloridine and that large inocula of these relatively resistant strains caused inactivation of the antibiotic. With cephalothin, in contrast, the MICs were invariably 2 μg/ml or less and there was little if any inactivation. It has subsequently been recommended by some authorities that cephaloridine be used in the treatment of severe staphylococcal infections only when susceptibility of the infecting strains can be reliably established in vitro (8). Regamey et al. (7) have recently reported that cefazolin is even more rapidly degraded by strains that inactivate cephaloridine. This study compares the relative susceptibility to inactivation of eight cephalosporins and further differentiates the characteristics of strains that cause marked destruction of cephaloridine from those that do not.

MATERIALS AND METHODS

Antibiotics. Standard laboratory powders were provided as follows: sodium cephapirin by Bristol Laboratories, Syracuse, N.Y.; cephradine by Squibb Institute for Medical Research, Princeton, N.J.; cefoxitin by Merck & Co., Rahway, N.J.; and sodium cephalothin, cephaloridine, sodium cefazolin, sodium cephalexin, and cefamandole lithium by Eli Lilly & Co., Indianapolis, Ind. Cephalothin and cefazolin disks (30 μg) were also supplied by Lilly.

Bacteria. One hundred recent clinical isolates of coagulase-positive, penicillinase-producing *S. aureus* were obtained from the Clinical Microbiology Laboratory of the University Hospital, Seattle, Wash. Standard disk susceptibility tests (1) were performed at 35 C by the Clinical Microbiology Laboratory, and all the strains were susceptible to methicillin and cephalothin.

Susceptibility studies. (i) Broth dilution tests. The MICs of cephaloridine and cephalothin for the 100 strains of *S. aureus* were determined in Mueller-Hinton Broth (MHB) and Trypticase soy broth (TSB) by adding 0.5 ml of 10^{-2} dilutions of overnight cultures to 0.5-ml amounts of serial twofold dilutions of the antibiotics in the same broths. The initial inoculum varied little from strain to strain, and between the broths, and averaged about 3×10^6 colony-forming units. The lowest concentration that suppressed visible growth after 18 h at 37 C was taken as the MIC. The MICs of six other cephalosporins (see above) were also determined against 10 of

the strains with MICs ≤ 2 μg/ml and 10 strains with MICs ≥ 8 μg/ml to cephaloridine in TSB. These strains included six with which more detailed inactivation studies were done, as described below.

(ii) Disk tests. An attempt was made with disk susceptibility tests to differentiate strains with cephaloridine MICs ≥ 8 μg/ml in TSB from those with MICs ≤ 2 μg/ml. Twenty-eight of the original 100 isolates were studied, together with 86 similar clinical isolates of *S. aureus*. Zone sizes with cephalothin and cefazolin disks (30 μg) were compared, using the standardized disk susceptibility test (1). MICs for cephaloridine in TSB were determined for all of the 114 strains.

(iii) Comparative antibiotic inactivation in broth cultures of growing organisms. Six strains with cephaloridine MICs ≥ 8 μg/ml and six with MICs ≤ 2 μg/ml in TSB, as determined by the broth dilution susceptibility tests described above, were studied for the eight cephalosporins. To each cephalosporin antibiotic (final concentration, 50 μg/ml) was added a 10^{-1} dilution of an overnight broth culture in a tube containing a final volume of 20 ml of MHB or TSB, and inactivation was studied during incubation at 37 C. At 0, 3, 6, 12, and 24 h, 0.1 ml was removed from each tube, appropriate dilutions were added to melted Trypticase soy agar for pour plates, and colonies were counted after an 18-h incubation. The initial colony counts (zero hours) averaged 2.8 × 10^7 in MHB and 3.8 × 10^7 in TSB; this was not a significant difference. At the same time intervals, 4 ml was removed from each tube for determination of the residual antibiotic concentration. Each sample was filtered through a disposable unit containing a 0.45-μm grid membrane (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and then frozen at −20 C until assayed. Controls, consisting of antibiotic in broth without bacteria, were run simultaneously to monitor spontaneous deterioration of the drugs. No difference was found in antibiotic concentrations between filtered and unfiltered broth.

Antibiotic inactivation by bacterial extracts. To determine whether different qualitative types of β-lactamases were produced by the two groups of staphylococci (cephaloridine-susceptible and -resistant strains, as differentiated by MICs in TSB), inactivation in broth by suspensions of equal amounts of powdered extracts of killed organisms was measured. Two strains from each group were grown in 200 ml of TSB containing low concentrations of cephalothin (0.05 to 0.1 μg/ml) to induce penicillinase production. After incubation overnight, the organisms were centrifuged at 3,000 rpm for 1 h, washed twice with acetone and once with ether, and allowed to dry (2). To equalize the quantity of extracts, 30 mg of dried powder from each strain was weighed out and resuspended in 20 ml of TSB containing 100 μg of cefazolin or cephaloridine per ml. Four-milliliter amounts were removed at intervals to determine antibiotic concentrations, using the procedure described above, except that the samples were not filtered, since the powders consisted of dead bacteria.

Antibiotic assay method. Concentrations of the antibiotics were measured by an agar well diffusion method in nutrient agar (Difco Laboratories, Detroit, Mich.), pH 7.2, with *Bacillus subtilis* ATCC 6633 (American Type Culture Collection, Rockville, Md.) as the indicator organism (4). Standard solutions of antibiotics were made at the time of each inactivation experiment and in the same type of broth (MHB or TSB) and were frozen at −20 C until the samples were assayed. Each determination was run in quintuplicate.

RESULTS

Susceptibility testing. (i) Broth dilution tests. The MICs of 100 strains of methicillin-susceptible, penicillinase-producing *S. aureus* (inoculum, 3 × 10^6) to cephaloridine in MHB were all 2 μg/ml or less (Fig. 1). When tested in TSB, however, as noted previously (2), 50% of strains had MICs of >2 μg/ml and 10% were >8 μg/ml. In contrast, cephalothin MICs were 2 μg/ml or less in both broths (Fig. 1). Ten cephaloridine-"susceptible" strains with MICs ≤ 2 μg/ml in TSB, and ten cephaloridine-"resistant" strains with MICs ≥ 8 μg/ml were also tested against six other cephalosporins with both broths, and results for the "resistant" strains are shown in Table 1. Cefazolin MICs were similar to those of cephaloridine, i.e., high in TSB and low in MHB, whereas cephalexin and cephradine, with less inherent activity against staphylococci, had high MICs (8 to 16 μg/ml) in both MHB and TSB. Cephapirin, cefamandole, and cefoxitin MICs were higher than that for cephalothin, due also to less inherent susceptibility, and a slight difference between the broths was observed with cephapirin. With the cephaloridine-susceptible strains (not shown) cephalexin, cephradine, and cefoxitin had high MICs (4 to 8 μg/ml) in both broths, whereas the MICs for the other

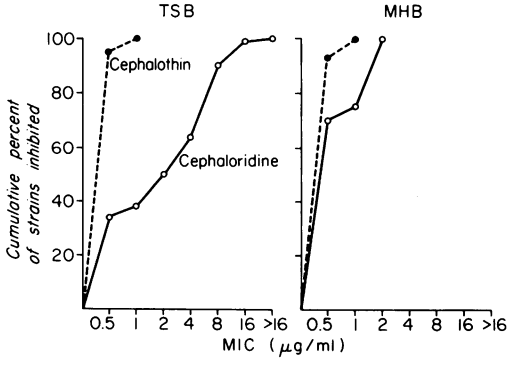

FIG. 1. *MICs of 100 strains of S. aureus, showing inhibition of all strains by ≤2 μg of cephalothin and cephaloridine per ml in MHB, whereas >2 μg of cephaloridine per ml was required for 50% of strains in TSB.*

cephalosporins were uniformly <2 μg/ml and mostly <1 μg/ml.

(ii) **Disk tests.** Of 114 strains, all of the 28 strains that had MICs ≥ 8 μg/ml for cephaloridine in TSB had zone sizes ≤ 26 mm with the cephalothin disk, and 26 of the 28 strains had zone sizes 4 mm smaller with the cefazolin than the cephalothin disk. However, an additional 35 strains that also had cephalothin zone sizes ≤ 26 mm had MICs of ≤4 μg/ml, and there was not a consistent 3- to 4-mm difference between the two disks.

On the other hand, of 72 strains with MICs ≤ 2 μg/ml, 47 had zone sizes ≥ 27 mm with the cephalothin disk, but with the other 25 strains the zone sizes were <27 mm. Thus, although there was a tendency for resistant strains to have cephalothin zones sizes ≤ 26 mm, as opposed to ≥27 mm for susceptible strains, enough exceptions occurred so that the disk test could not be relied on the separate resistant from susceptible strains.

(iii) **Comparative antibiotic inactivation.** Figure 2 depicts the average inactivation in TSB by six resistant strains of *S. aureus* (MIC for cephaloridine ≥8 μg/ml in TSB) for all eight cephalosporins (initial antibiotic concentration, 50 μg/ml). Cefazolin was the most rapidly inactivated by the bacteria (total degradation minus spontaneous degradation), 93% in 6 h

TABLE 1. *MICs (μg/ml) of 10 resistant strains showing striking differences between the two broths (MHB and TSB), especially with cephalothin as compared with cephaloridine and cefazolin*

Strain no.	Cephalothin		Cephaloridine		Cefazolin		Cephalexin		Cephradine		Cephapirin		Cefamandole		Cefoxitin	
	MHB	TSB	MHB	TSB	MHB	TSB	MHB	TSB	MHB	TSB	MHB	TSB	MHB	TSB	MHB	TSB
1	<1	<1	1	16	2	8	16	16	16	4	1	2	2	2	4	4
2	<1	<1	2	16	2	16	16	16	16	4	1	2	4	2	4	4
3	<1	<1	2	16	2	16	16	8	16	8	1	4	2	2	8	4
4	<1	<1	2	16	<1	8	8	8	8	8	1	2	2	2	4	4
5	<1	<1	2	16	2	8	16	8	8	4	1	2	2	4	8	4
6	<1	<1	1	8	1	8	8	8	16	16	1	2	2	2	8	4
7	<1	<1	1	8	<1	8	8	8	8	8	1	1	4	2	4	4
8	<1	<1	2	16	1	16	8	8	16	16	1	4	2	4	8	4
9	<1	<1	2	16	1	16	8	8	8	16	1	2	2	4	4	4
10	<1	<1	4	16	4	>16	16	16	32	32	1	1	2	2	4	4

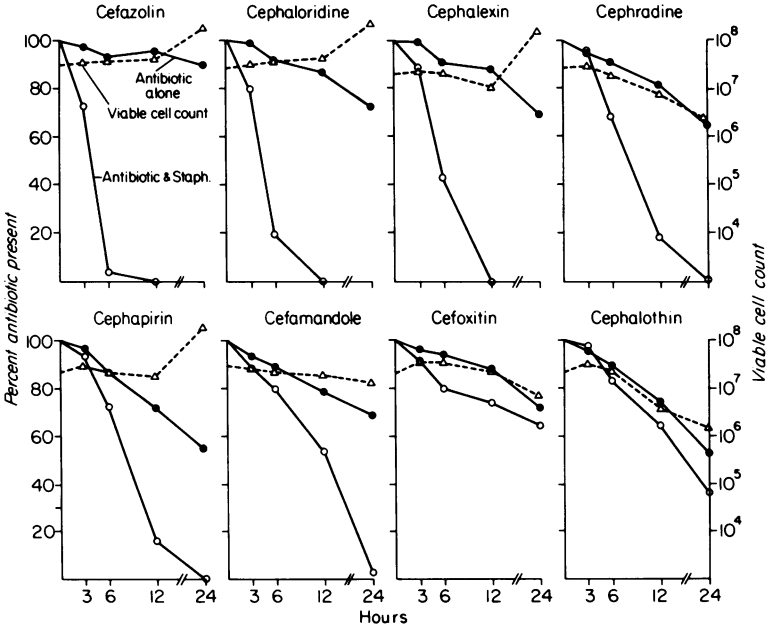

FIG. 2. *Average inactivation of eight cephalosporins and growth curves for six resistant strains of S. aureus (MICs ≥8 μg/ml for cephaloridine in TSB). The initial antibiotic concentrations (100%) were 50 μg/ml.*

versus 70% for cephaloridine (paired sample t test: $P < 0.01$), and no antibacterial activity remained for either at 12 h. This occurred despite the fact that spontaneous degradation in broth alone was greater for cephaloridine (27 versus 10% at 24 h). Inactivation of cephalexin was slightly less than that of cephaloridine, and cephradine was degraded more slowly than cephalexin, with 18% of the original concentration being measurable at 12 h. Cephapirin was not significantly different from cephradine, but cefamandole was less inactivated than cephapirin, with 54 versus 16% being present at 12 h. Cefoxitin cephalothin underwent little, if any, inactivation as compared to the controls.

In contrast, the pattern of inactivation was completely different for the susceptible organisms (MIC for cephaloridine ≤ 2 μg/ml in TSB). Only cefazolin and cephaloridine showed any significant inactivation as compared with the broth control, and there was much less inactivation than with the resistant strains. Furthermore, with all six strains cephaloridine underwent greater bacterial inactivation than cefazolin, 66 versus 14% at 12 h ($P < 0.01$), the reverse of what occurred with the resistant strains. (Fig. 3).

When these studies of the resistant and susceptible strains were repeated in MHB the comparative order and relative rates of inactivation were similar, but the degree of inactivation was much less than in TSB.

Correlation of colony counts with inactivation. With the large inoculum used for the inactivation studies (2.8×10^7 to 3.8×10^7), there was an average decrease in colony-forming units of only about 1 log with cephalothin in 24 h with the resistant strains in TSB (Fig. 2). Cefoxitin, which also underwent little if any inactivation, showed an even smaller decline in colony counts, due possibly to its considerbly higher MICs (Table 1). Where there was marked inactivation during the first 12 h (cefazolin, cephaloridine, cephalexin, and cephapirin) the colony counts showed no initial decline, and there was a significant increase after 12 h. Cephradine was an exception; the bactericidal action was similar to that of cephalothin although inactivation was comparable to that of cephapirin.

Despite similar initial colony counts, the bactericidal effects of all the antibiotics were more marked in MHB than in TSB. In this broth cephalothin and cefoxitin produced the greatest killing, with an average of 2 to 2.5 log decrease in viable cell count at 24 h. With cefazolin, cephapirin, and cefamandole there was moderate killing in the first 12 h, but subsequent increase in colony counts.

Antibiotic inactivation by bacterial extracts. With equal amounts of powdered extracts of the staphylococci, the comparative rates of inactivation of the cephalosporins were the same as those observed with the living organisms. Extracted powder (rich in β-lactamase) from the susceptible strains produced greater destruction of cephaloridine than cefazolin, whereas extracts from the resistant strains inactivated cefazolin more than cephaloridine. Thus, these observations confirmed the impression that qualitatively different β-lactamases were produced by the susceptible and resistant strains.

DISCUSSION

These studies confirm earlier observations that cephaloridine is quite rapidly inactivated by strains of *S. aureus* that have high MICs for

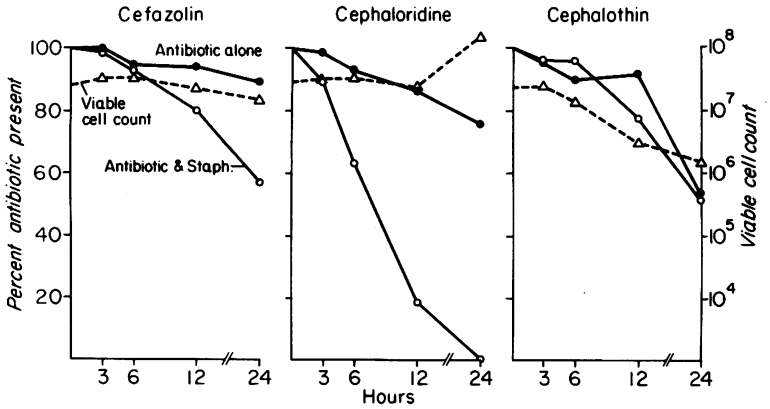

FIG. 3. *Average inactivation of three cephalosporins and growth curves for six susceptible strains (MICs ≤2 μg/ml for cephaloridine in TSB). The pattern of inactivation was the reverse of that observed with resistant strains and was due to qualitatively different β-lactamases.*

this antibiotic (2), and the more recent demonstration that cefazolin is even more susceptible to inactivation by these strains (7). Only cephalothin and cefoxitin seemed highly resistant to inactivation. The comparative order of decreasing resistance to inactivation of the six other cephalosporins tested was as follows: cefamandole, cephapirin, cephradine, cephalexin, cephaloridine, and cefazolin. Quinn et al. have also observed a number of strains of *S. aureus* with high MICs for cephaloridine and cefazolin and noted that a strain from a case of endocarditis caused marked inactivation of cefazolin (6). Sabath et al. (9) found cephaloridine to be more susceptible to inactivation than cefazolin but, since differentiation between strains with high and low MICs was not a part of their study, the results cannot be considered comparable.

As in the earlier paper by Benner et al. (2), all of 100 strains of penicillin G-resistant *S. aureus* in the present study were inhibited by 2 μg or less of cephalothin per ml, but only 50% were inhibited by this concentration of cephaloridine. A new observation in the present studies was the fact that strains causing marked inactivation of cephaloridine had high MICs in TSB but not in MHB. (Table 1). Thus, MICs greater than 2 μg/ml were not observed when the broth dilution tests were done only in MHB. The higher MICs in TSB, which contains dextrose, were probably due to a greater production of β-lactamase in this broth, as demonstrated in the inactivation studies. Since there was not a clear-cut difference in the inoculum size, a greater production of β-lactamase by each colony-forming unit in TSB seemed responsible for the difference between the broths. Cefazolin MICs were similar to those of cephaloridine, namely, high in TSB but not in MHB. Cephalexin and cephradine had MICs well above 2 μg/ml in MHB as well as TSB (Table 1), due mainly to less inherent susceptibility, with inactivation a contributing factor.

Another point of interest was the fact that with susceptible strains of *S. aureus*, i.e., those with MICs ≤ 2 μg/ml for cephaloridine in TSB as well as MHB, there was much less total inactivation and it was always greater with cephaloridine than with cefazolin, exactly the reverse of what occurred with the resistant strains. A careful comparison of equal amounts of powdered extracts of the two groups of organisms showed that there was an actual qualitative difference in the β-lactamases produced by the susceptible and resistant strains. Thus, the observation of less resistance to β-lactamase with cephaloridine compared to cefazolin in the study by Sabath et al. (9) could have been due to a preponderance of susceptible strains.

Attempts using disk susceptibility testing to differentiate strains with cephaloridine MICs ≥ 8 μg/ml from those that were ≤2 μg/ml in TSB met with limited success. There was a tendency for strains with high MICs to have zone sizes of ≤26 mm with the cephalothin disk and for the zones to be up to 4 mm smaller with the cefazolin disk. Also, only 4 of 51 strains with zone sizes ≥ 27 mm with the cephalothin disk had cephaloridine MICs > 2 μg/ml. However, inconsistencies occurred with sufficient frequency so that predictions based on zone sizes were not reliable. Thus, an MIC of ≥8 μg/ml for cephaloridine in TSB, with an inoculum ≥10^6 colony-forming units, remains the only practical way of detecting strains with a marked capacity for inactivating cephaloridine and cefazolin.

The possible clinical significance of these observations is difficult to assess. The fact that 50 μg of cefazolin per ml, the cephalosporin most susceptible to β-lactamase, was almost completely inactivated within 6 h by a large inoculum of staphylococci in a broth culture (Fig. 2) suggests that it might be difficult to maintain an adequate concentration of this antibiotic in the patient even with repeated doses. Possible clinical failures, i.e., persistently positive blood cultures after several days of therapy, have been reported for both cephaloridine (5) and cefazolin (6) in staphylococcal endocarditis. On the other hand, Benner et al. (2) were unable to detect inadequate responses to cephaloridine in patients with severe staphylococcal (non-endocarditis) infections caused by strains with high MICs for this antibiotic. The safest policy at present might be to follow the suggestion that patients with severe staphylococcal infections be treated with cephalothin unless it can be demonstrated that the infecting organism is clearly sensitive (8), i.e., that the MIC is not ≥8 μg/ml in TSB with cephaloridine or cefazolin.

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